(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 334 358**
A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **89105251.6**

(22) Date of filing: **23.03.89**

(51) Int. Cl.[4]: **C12N 9/80 , C12P 13/06 , C12N 1/20 , //(C12P13/06, C12R1:06)**

The applicant has filed a statement in accordance with Rule 28 (4) EPC (issue of a sample only to an expert). Accession numbers of the deposits: FERM BP - 1649 and FERM BP - 1773

(30) Priority: **24.03.88 JP 70217/88**

(43) Date of publication of application:
27.09.89 Bulletin 89/39

(84) Designated Contracting States:
DE FR GB

(71) Applicant: **KYOWA HAKKO KOGYO CO., LTD.**
6-1, Ohte-Machi Itchome
Chiyoda-ku Tokyo(JP)

(72) Inventor: **Ozaki, Akio**
2-2-104, Kyowa-cho
Hofu-shi Yamaguchi-ken(JP)
Inventor: **Kawasaki, Hideki**
1-2, Kyowa-cho
Hofu-shi Yamaguchi-ken(JP)
Inventor: **Hashimoto, Yukio**
2-2-102, Kyowa-cho
Hofu-shi Yamaguchi-ken(JP)
Inventor: **Tamura, Keishiro**
1422-66, Oaza Tajima
Hofu-shi Yamaguchi-ken(JP)
Inventor: **Ochiai, Keiko**
2-2-3-27, Sobudaidanchi
Sagamihara-shi Kanagawa-ken(JP)
Inventor: **Kawamoto, Isao**
1-21-2, Fujimino
Hiratsuka-shi Kanagawa-ken(JP)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4 P.O. Box 86 07 67
D-8000 München 86(DE)

(54) Novel D-amidase and process for producing D-alpha-alanine and/or L-alpha-alanineamide.

(57) A novel D-amidase is described. The enzyme specifically hydrolyzes D-α-alanineamide into D-α-alanine. It is produced by culturing a microorganism belonging to the genus Arthrobacter, and is useful as an enzyme for efficiently producing D-α-alanine having a high optical purity and/or L-α-alanineamide from DL-α-alanineamide or D-α-alanineamide at low cost.

EP 0 334 358 A2

NOVEL D-AMIDASE AND PROCESS FOR PRODUCING D-α-ALANINE AND/OR L-α-ALANINEAMIDE

Background of the Invention

The present invention relates to a novel enzyme capable of specifically hydrolyzing D-α-alanineamide to produce D-α-alanine (hereinafter referred to as D-amidase) and a process for producing D-α-alanine and/or L-α-alanineamide using the enzyme.

D-α-alanine is used as a sweetening compound or as a synthetic intermediate or starting compound for synthesizing various physiologically active substances. L-α-Alanineamide is a starting compound for producing L-α-alanine, which is an important amino acid in the food and pharmaceutical industry.

The following processes for producing D-α-alanine have been known so far.

(1) A microbiological process for producing D-α-alanine though direct fermentation [Japanese Published Unexamined Patent Application Nos. 22881/76 and 76482/77].

(2) A process for producing D-α-alanine by separating D-α-alanine from DL-α-alanine through the action of microorganism having the ability to decompose only L-α-alanine [Ohshima and Tanaka: Amino Acid Nucleic Acid, 15, 89-93 (1966)].

(3) A process for producing D-α-alanine by treating the N-acyl compound of DL-α-alanine with an acylase produced by a microorganism and by successive optical resolution of DL-α-alanine (Japanese Published Examined Patent Application No. 22380/66).

(4) A process for producing D-α-alanine by treating 5-methylhydantoin with microorganisms having a hydantoinase activity to form D-(N-carbamoyl)alanine, and by treating D-(N-carbamoyl)alanine chemically or microbiologically [Yamada, et al: Hakko To Kogyo, 38, 937 (1980), Japanese Published Unexamined Patent Application Nos. 91189/78, 89088/79,88697/80, 104890/80, 114291/80, etc.]

(5) A process for producing D-α-alanine by hydrolyzing D-α-alanineamide through the hydrolytic activity possessed by microorganisms belonging to the genus Bacillus, Bacterium, Micrococcus, Brevibacterium, Achromobacter, Alcaligenes, Kurthia, Pseudomonas, Rhodococcus or Serratia [Japanese Published National Publication No. 500319/81, Japanese Published Unexamined Patent Application Nos. 184392/85, 96989/86 and 274690/86].

(6) A process for producing a D-α-amino acid by hydrolyzing DL-α-amino acid amide through the hydrolytic activity specific to D-α-amino acid amide and possessed by microorganisms belonging to the genus Rhodococcus [Japanese Published Unexamined Patent Application No. 87998/88].

(7) A process for producing D-α-alanine by treating pyruvic acid with a D-amino-acid transaminase [Japanese Published Unexamined Patent Application No. 205790/87].

(8) A process for producing D-α-alanine by subjecting DL-α-alanine p-chlorobenzenesulfonate to preferential crystallization in order to achieve chemical optical resolution [Japanese Published Examined Patent Application No. 14369/72 and Japanese Published Unexamined Patent Application No. 57914/73].

Among the aforementioned processes for producing D-α-alanine, the processes (1), (2), (6) and (8) produce no or little D-α-alanine, and the processes (3), (4) and (7) are complicated because they consist of several steps. The processes (5) and (7) require an expensive optically active substrate and thus raise the cost of production.

The enzymes capable of hydrolyzing D-α-alanineamide are disclosed in the Abstracts of the 1988th Meeting of the Agricultural Chemical Society of Japan, page 352.

No process for producing L-α-alanineamide of high optical purity at an industrial scale at low cost is known as yet.

It is therefore the object of the present invention to provide an enzyme capable of producing D-α-alanine and L-α-alanineamide of high optical purity directly from inexpensive DL-α-alanineamide and a process for producing D-α-alanine and L-α-alanineamide using the enzyme.

Extensive studies have been made on the development of an industrially favourable process for producing D-α-alanine from DL-α-alanineamide. As a result, the present inventors have found that microorganisms belonging to the genus Arthrobacter produce an enzyme capable of specifically hydrolyzing D-α-alanineamide thereby producing D-α-alanine from DL-α-alanineamide or D-α-alanineamide. Isolation and purification of the enzyme and examination of its physicochemical properties reveals that the enzyme is novel, and the present invention has thus been accomplished.

Summary of the Invention

The present invention provides a novel D-amidase as well as a process for producing D-α-alanine and/or L-α-alanineamide, which comprises carrying out an enzymatic hydrolysis in an aqueous medium containing DL-α-alanineamide or D-α-alanineamide in the presence of a culture or cells of a microorganism belonging to the genus Arthrobacter and being capable of producing D-amidase or a treated product thereof or D-amidase isolated therefrom, and recovering D-α-alanine and/or L-α-alanineamide from the resulting reaction mixture.

The physicochemical properties of the novel D-amidase are given below.

1) Activity and substrate specificity:

It specifically hydrolyzes D-α-alanineamide into D-α-alanine; its hydrolytic activity in respect of L-α-alanineamide is 0 to 1.5% of that in respect of D-α-alanineamide.

2) Optimum pH:

pH 7 to 8 at 30 °C

3) Optimum temperature:

40 to 45 °C at a pH of 7.5

4) Heat stability:

It is inactivated when allowed to stand at a temperature above 60 °C for 10 minutes.

5) pH stability:

It is stable within a range of pH 6.5 to 10 at 30 °C.

6) Molecular weight:

50,000 ± 5,000 (SDS-polyacrylamide electrophoresis)

7) Activation:

No coenzyme is required for activation.

8) Isoelectric point:

pH 5.2 ± 0.3

Brief Description of the Drawings

Fig. 1 shows the relative activity in dependency of the pH, the enzyme activity at pH 7.5 being defined as 100%.

Fig. 2 shows the relative activity in dependency of the temperature, the enzyme activity at 37 °C being defined as 100%.

Fig. 3 shows the pH stability of the enzyme.

Fig. 4 shows the heat stability of the enzyme.

Description of the Invention

Any microorganisms can be used for producing the enzyme of the present invention, as long as they belong to the genus Arthrobacter and have the ability to produce the enzyme having the properties described above. An example is Arthrobacter sp. H-4904.

Arthrobacter sp. H-4904 is a microorganism newly isolated from the natural source.

Bacteriological properties of Arthrobacter sp. H-4904 are described below:

(a) Morphology

1) Form and size of the cells:

Spherical form (0.8 - 1.0 μm in diameter) and rod form (0.8 μm in diameter and 1.2 - 1.5 μm long).

2) Motility: Motile with flagella occurring in polar positions.

3) Spore: none

4) Gram staining: positive

5) Acid resistance: scarcely observed

(b) Growth particulars on various media

1) Cells growing on bouillon-agar form circular, convex, smooth colonies, showing an opaque, whitish yellow color without formation of diffusible pigments.

2) Cells grow well on bouillon-agar slant culture and show an opaque, whitish yellow color.

3) Cells growing in bouillon liquid culture impart a turbid appearance without formation of a surface membrane.

4) Cells growing in bouillon-gelatin stab culture do not lead to liquefaction

5) Litmus milk: no reduction of litmus and no coagulation.

(c) Physiological properties:

1) Nitrate reduction: positive

2) Denitrification reaction: positive

3) MR test: negative

4) Voges-Proskaner-test: negative

5) Indole formation: negative

6) Hydrogen sulfide formation: negative

7) Starch hydrolysis: negative

8) Citric acid utilization (Simons' medium): positive

9) Utilization of inorganic nitrogen source

Nitrate: negative

Ammonium salt: weakly positive

10) Pigment formation: negative

11) Urease: negative

12) Oxidase: negative

13) Catalase: positive

14) Growth range

1) pH: 5.0 - 9.0 (optimum pH: 6.0 - 8.0)

2) Temperature: 15 - 37° C (optimum temperature: 30° C)

15) Oxygen relationship: aerobe or facultative anaerobe

16) OF test: negative

17) Acid and gas formation from saccharides:

	Acid	Gas (peptone-water)
L-arabinose	-	-
D-xylose	-	-
D-glucose	-	-
D-mannose	-	-
D-fructose	-	-
D-galactose	-	-
Maltose	-	-
Sucrose	-	-
Lactose	-	-
Trehalose	-	-
D-sorbitol	-	-
D-mannitol	-	-
Inositol	-	-
Glycerine	-	-
Starch	-	-

18) Sodium chloride resistance: grow at 15% NaCl.

(d) Chemical Composition

1) Peptidoglycan-constituting amino acids: lysine, alanine and glutamic acid
2) mol% G + C (Tm): 63.17

The microorganism with the aforementioned bacteriological properties was identified as a bacterium belonging to the genus Arthrobacter, referring to Bergey's Manual of Systematic Bacteriology, Vol.2 (1986) on the basis of such properties as gram-positive, spherical or rod form; motile with flagella occurring in polar positions, aerobe or facultative anaerobe; non-formation of spores; lysine, alanine and glutamic acid as peptidoglycan-constituting amino acids; mol% G + C of DNA being 63.17. The strain was named Arthrobacter sp. H-4904 and has been deposited with the Fermentation Research Institute (FRI), the Agency of Industrial Science and Technology under the Budapest Treaty on January 14, 1988 with the accession number of FERM BP-1649.

Any natural or synthetic medium can be used as the medium for culturing the microorganism, as long as they contain a carbon source, a nitrogen source, inorganic salts, etc. in appropriate amounts that can be assimilated by the microorganism and the microorganism having an ability to produce the enzyme of the present invention can be efficiently cultured therein.

As the carbon source, carbohydrates such as glucose, sucrose, molasses, starch hydrolyzate, etc., organic acids such as acetic acid, propionic acid, etc., and alcohols such aas ethanol, propanol, etc. can be used.

As the nitrogen source, ammonia, various ammonium salts of inorganic and organic acids such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, etc., amines and other nitrogen-containing compounds, peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, soybean cake hydrolyzate, various fermentation cells and their digested products, etc. can be used.

As the inorganic materials, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, etc. can be used.

Culturing is carried out under aerobic conditions with shaking, submerged aeration stirring, etc. preferably at a temperature of 15 to 37°C, usually for 16 - 72 hours, while keeping the pH at 5.0 - 9.0 by adding an inorganic or organic acid, an alkaline solution, urea, calcium carbonate, ammonia, etc.

For the purpose of inducing and accumulating D-amidase it is necessary to add 0.1 - 20 g/l D-α-alanineamide, L-α-alanineamide or DL-α-alanineamide to the culture medium.

By the use of an appropriate mutant, D-amidase can be accumulated without adding D-α-alanineamide, L-α-alanineamide or DL-α-alanineamide (which is hereinafter referred to as "inducing substance") to the medium.

The mutant can be obtained from Arthrobacter sp. H-4904 as the parent strain according to the ordinary mutagenesis, for example, through ultraviolet irradiation, radiation irradiation such as X-ray irradiation, treatment with mutation-inducing agent, etc.

After the mutation treatment, a desired mutant can be obtained by recovering colonies growing in an ordinary nutrient medium, for example, a bouillon-yeast extract medium and selecting a strain that accumulates D-amidase in a medium containing no inducing substance.

An example of the thus obtained mutant is Arthrobacter sp. H-7095, which has been deposited with the FRI under the Budapest Treaty since March 2, 1988 with the accession number of FERM BP-1773.

In order to recover and purify the enzyme from the culture, conventional purification methods for an enzyme may be used. For example, the culture is centrifuged and the cells are collected. The collected cells are disrupted by means of mechanical disruption using DYNO-MILL, French Press, Manton-Gaulin homogenizer, ultrasonication, etc. The resulting suspension is centrifuged to remove cell debris and the enzyme in the supernatant is isolated and purified by a salting-out method using ammonium sulfate and ion exchange chromatography using DEAE-Sepharose, CM-Sepharose, etc. Thereby, an enzyme which is pure according to SDS-polyacrylamide gel electrophoresis, can be obtained.

The enzyme activity is determined as follows.

1.0 mℓ of 50 mM phosphate buffer (pH 7.5) containing 250 mM D-α-alanineamide is heated at 30°C for 5 minutes, then 0.1 mℓ of an enzyme solution is added thereto and the mixture is incubated at 30°C for 30 minutes. The reaction is terminated by adding 0.1 mℓ of 6N hydrochloric acid thereto. The amount of the D-α-alanine formed during the reaction is determined by high performance liquid chromatography (HPLC) under the following conditions.

Column: CHIRALPAK WE(-), manufactured by Daicel Ltd.
Eluate: 0.25 mM $CuSO_4$ aqueous solution
Flow rate: 1 mℓ/min
Column temperature: 45°C

Method for detection:
o-Phthalaldehyde is added to react at 50°C and fluorescence is detected (excitation wavelength: 344 nm; fluorescent wavelength: 444 nm)

The enzyme activity is given in units, 1 unit (U) corresponding to the activity necessary to hydrolyze D-α-alanineamide to 1 μmol of D-α-alanine in one minute.

The activity for hydrolyzing L-α-alanineamide to L-α-alanine (hereafter referred to as L-amidase activity) can be determined under the conditions described above except that L-α-alanineamide is used in place of D-α-alanineamide.

Though D-α-alanine and/or L-α-alanineamide can be obtained by treating DL-α-alanineamide or D-α-alanineamide with the enzyme of the present invention, it is preferred that an enzymatic hydrolysis is carried out in an aqueous medium containing DL-α-alanineamide or D-α-alanineamide in the presence of a culture or cells of the microorganism belonging to the genus Arthrobacter and being capable of producing the enzyme of the present invention or a treated product thereof, and that D-α-alanine and/or L-α-alanineamide can be recovered from the resulting reaction mixture.

The enzymatic hydrolysis can be carried out either during the cultivation of the microorganism or by allowing the culture, cells, the treated product thereof, or the purified enzyme to react on DL-α-alanineamide or D-α-alanineamide in an aqueous medium after the cultivation has been finished.

The above-mentioned treated product of cells of the microorganism having the D-amidase activity includes, for example, dried cells, freeze-dried cells, surfactant-treated cells, enzyme-treated cells, ultrasonically disrupted cells, mechanically disrupted cells, solvent-treated cells, and proteinous fractions of cells. It further includes immobilized products of cells and the aforesaid treated products thereof. Purified D-amidase may be used and may also be used as the immobilized products.

The aqueous medium includes, for example, water, buffer solutions of phosphate, carbonate, acetate, borate, citrate, Tris, etc., alcohols such as methanol, ethanol, propanol, etc., esters such as ethyl acetate, etc.. ketones such as acetone, etc., and amides such as acetamide, etc.

When purified enzyme is used in the reaction, 5 to 50% glycerol are preferably added to the aqueous medium in order to improve the stability of the enzyme.

The reaction is usually carried out at a temperature of 15 to 50°C and a pH of 6.0 to 9.5 for 1 to 72 hours. The potency of enzyme in the reaction mixture depends on the amount of DL-α-alanineamide or D-α-alanineamide used and on the reaction time. It usually is 1 to 300 kU/ℓ.

When cells are used in the reaction, the concentration of cells is usually 1 to 50 g/ℓ as wet cells. The DL-α-alanineamide or D-α-alanineamide for use in the reaction can be in the free form, hydrochloride form or the sulfate form. In case of DL-α-alanineamide, 1 - 500 g/ℓ, preferably 1 - 400 g/ℓ can be used, and in case of D-α-alanineamide, 1 - 300 g/ℓ, preferably 1 - 200 g/ℓ can be used.

The alanine racemase usually contained in microbial cells is an enzyme that catalyzes racemation of optically active alanine and lowers the optical purity of D-α-alanine formed in the process of the present invention. The microorganism to be used in the present invention has a smaller alanine racemase content, and thus D-α-alanine of satisfactorily optical purity can be obtained by use of the microorganisms of the present invention. However, D-α-alanine of much higher optical purity can be obtained by appropriately using well known procedures for suppressing the alanine racemase activity, for example, by obtaining a mutant with no or little alanine racemase activity through an ordinary mutagenesis [J. Wild, et al.: Mol. Gen. Genet. 198, 315-322 (1985)]; by inactivating the activity of alanine racemase in the microorganism through a heat treatment, etc. [Takamatsu, Tosa and Chihata: J. Jap. Chem. Soc. 9 1369 (1983)], and by adding an alanine racemase inhibitor to the aqueous medium during the reaction [Kagaku To Seibutsu 20 770-772 (1986); Seikagaku Jikken Koza 11 275-296].

When D-α-alanineamide is used in the enzymatic hydrolysis, D-α-alanine is formed and accumulated in the aqueous medium and concurrently, L-α-alanineamide remains in the aqueous medium after the hydrolysis. Thus, L-α-alanineamide can be obtained by recovering it from the aqueous medium.

D-α-alanine and L-α-alanineamide can be recovered from the culture or the aqueous medium according to a usual separation procedure, for example, through column chromatography using ion exchange resin, etc., or through crystallization.

The present invention is described in the following Examples.

Example 1

150 mℓ of BYG medium [a medium containing 2% of bouillon powder (product of Kyokuto Co.), 0.5% of yeast extract (product of Difco), 0.5% of polypeptone and 0.2% of glucose, adjusted to pH 7.2 with 6N

NaOH] was poured into each of 2 ℓ-flasks provided with baffles, and sterilized at 120°C for 20 minutes. Then, each of the media was inoculated with one loopful of Arthrobacter sp. H-4904 grown on a bouillon slant and cultivated at 30°C for 20 hours with shaking. The resulting culture was used as a seed culture.

Separately, a medium containing 3% of glucose, 2% of corn steep liquor, 0.5% of peptone, 1% of NaCl, 2% of $(NH_4)_2SO_4$, 0.3% of $MgSO_4 \cdot 7H_2O$, 0.001% of $FeSO_4 \cdot 7H_2O$, 0.0001% of $MnSO_4 \cdot 7H_2O$ and 0.6% of DL-α-alanineamide and having a pH of 7.2 was prepared, and 18ℓ of the thus prepared medium was poured into a 30 ℓ-jar fermenter and sterilized at 120°C for 20 minutes. Then, the medium was aseptically inoculated with 2 l of the seed culture and cultivated under agitation and aeration (450 rpm, 10 ℓ/min) at 30°C for 30 hours. The D-amidase activity of the thus obtained culture was 64 U/mℓ.

The cells obtained by centrifuging the culture were suspended in 1.9 ℓ of 50 mM sodium phosphate buffer (pH 7.5). Then, the cells were disrupted with DYNO-MILL (laboratory mill model KDL, manufactured by W.A. Bachafen Maschinenfabrik). The cell suspension was centrifuged and the resulting supernatant was loaded onto a DEAE-Sepharose Fast Flow (manufactured by Pharmacia Inc.) column which had been equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5). Then, density gradient elution was carried out using the same buffer in which the concentration of sodium chloride was increased from 0 to 0.4 M. D-amidase was eluted in the fraction containing 0.2 M sodium chloride. The active fraction was further loaded onto a BUTYL TOYOPEARL (TSK-GEL 650C, manufactured by Toyo Soda Mfg. Co., Ltd.) column which had been equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 20% saturated ammonium sulfate. Then, density gradient elution was carried out using the same buffer in which the saturated ammonium sulfate was decreased from 20% to 0.1%. D-amidase was eluted in the 15 to 10% saturated ammonium sulfate fractions. After desalting the active fraction with a UF membrane (SIP-1013, manufactured by Asahi Chemical Co., Ltd.), glycerol was added to the active fraction in a concentration of 25% (v/v). The fraction was then loaded onto a DEAE-Trisacryl LS (manufactured by Réactifs IBF Soc. Chim) column which had been equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 25% (v/v) glycerol. Then, density gradient elution was carried out using the same buffer in which the sodium chloride was increased from 0 to 0.4 M. D-amidase was eluted in the fractions containing 0.2 M sodium chloride.

The D-amidase activity of the thus obtained enzyme was 6.0×10^4U. The enzyme showed a single band in SDS-polyacrylamide gel electrophoresis corresponding to a molecular weight of about 50,000.

The specific activity and the Km value of the enzyme with D-α-alanineamide and L-α-alanineamide as substrates are shown in Table 1.

Table 1

	D-α-Alanineamide	L-α-Alanineamide
Specific Activity (U/mg protein)	1800	17.4
Km (mM)	4.2	26.1

In Fig. 1, the activity in dependency of the pH is shown as relative activity, the enzyme activity at pH 7.5 being defined as 100%. The enzyme had the optimum pH of 7 to 8 at 30°C.

In Fig. 2, the activity in dependency of the temperature is shown as relative activity, the enzyme activity at 37°C being defined as 100%. As shown in Fig. 2, the enzyme has an optimum temperature of 40 to 45°C at pH 7.5.

The pH stability of the enzyme was determined by the following method.

To 0.95 mℓ of various buffer solutions having a pH of about 6 to 10 containing 25% glycerol, 0.05 mℓ of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 19.2 U of the enzyme was added. The mixtures were allowed to stand at 30°C for 2 hours. Then, 0.05 mℓ of each enzyme solution was added to 0.5 mℓ of D-α-alanineamide solution [50 mM phosphate buffer (pH 7.5) containing 12.5 g/ℓ of D-α-alanineamide and 25% (v/v) glycerol], incubated at 37°C for 30 minutes, and the reaction was terminated by the addition of 0.1 mℓ of 6N hydrochloric acid. The amount of D-α-alanine formed during the incubation was quantitatively determined. The enzyme activity was expressed as relative activity, the enzyme activity before the incubation at 30°C for 2 hours being defined as 100%. The results are shown in Fig. 3. As shown in Fig. 3, the enzyme was stable in a pH range of 6.5 to 10.

The temperature stability of the enzyme was determined by the following method.

A solution of 192 U of the enzyme in 0.5 ml 50mM Tris-hydrochloride buffer (pH 7.5) was diluted 10-fold with 50 mM phosphate buffer (pH 7.5) containing 25% glycerol. 0.5 ml of the diluted solution were allowed to stand for 10 minutes at various temperature, and immediately thereafter ice-cooled. To 0.5 mℓ of D-α-alanineamide solution [50 mM phosphate buffer (pH 7.5) containing 12.5 g/ℓ of D-α-alanineamide and

25% (v/v) glycerol, 0.05 mℓ of the enzyme solution was added, incubated at 37°C for 30 minutes, and the reaction was terminated by the addition of 0.1 mℓ of 6N hydrochloric acid. The amount of D-α-alanine formed during the incubation was quantitatively determined. The enzyme activity was expressed as relative activity, defining the enzyme activity before the incubation at various temperatures for 10 minutes as 100%. The results are shown in Fig. 4. As shown in Fig. 4, the enzyme was inactivated when allowed to stand for 10 minutes at temperatures above 60°C.

Example 2

BYG medium (150 mℓ) having the same composition as in Example 1 was poured into each of 2 ℓ-flasks provided with baffles, and sterilized at 120°C for 20 minutes. Then, each of the media was inoculated with one loopful of Arthrobacter sp. H-4904 grown on a bouillon slant and cultivated at 30°C for 20 hours with shaking. The resulting culture was used as a seed culture.

Separately, a medium containing 3% of glucose, 2% of corn steep liquor, 0.5% of peptone, 1% of NaCl, 2% of $(NH_4)_2SO_4$, 0.3% of $MgSO_4 \cdot 7H_2O$, 0.001% of $FeSO_4 \cdot 7H_2O$, 0.0001% of $MnSO_4 \cdot 7H_2O$ and 0.6% of DL-α-alanineamide and having a pH of 7.2 was prepared, and 1.5 ℓ of the thus prepared medium was poured into a 3 ℓ-jar fermenter and sterilized at 120°C for 20 minutes. Then, the medium was aseptically inoculated with 150 ml of the seed culture and cultivated under agitation and aeration (800 rpm, 1 vvm) at 30°C for 24 hours. The thus obtained culture was centrifuged at 4°C for 10 minutes at 5,000 rpm. A solution containing 420 g of DL-α-alanineamide (592 g of hydrochloride thereof), 15.6 g of $NaH_2PO_4 \cdot 2H_2O$ and 35.8 g of $Na_2HPO_4 \cdot 12H_2O$ in deionized water was added to 10 g of the thus obtained wet cells to reach a total volume of 2 ℓ. The pH of the resulting mixture was adjusted to 6.7 with 10N NaOH. The hydrolysis reaction was carried out at 38°C for 10 hours with gentle stirring. The mixture was kept at pH 6.7 with 6N HCl during the reaction.

After the hydrolysis reaction had been finished, the amount of D-α-alanine and the amount of L-α-alanineamide in the reaction mixture were quantitatively determined. The results are shown in Table 2.

Table 2

	Titer (g/ℓ)	Reaction yield (%)	Optical purity (%)
D-α-alanine	105	99	99.3
L-α-alanineamide	105	-	99.0

The pH of 1 ℓ of the reaction mixture was adjusted to 4.5 and the mixture passed through a column packed with 3 ℓ of Diaion SKIB (NH_4^+ form) (product of Mitsubishi Kasei Corporation) to separate D-α-alanine from L-α-alanineamide. The respective fractions were crystallized by condensation under reduced pressure whereby 85 g of D-α-alanine (optical purity: 99.5% or higher) and 75 g of L-α-alanineamide (optical purity: 99.5% or higher) were obtained.

Example 3

The hydrolysis reaction was carried out in the same manner as in Example 2 except that 210 g of D-α-alanineamide was used in place of DL-α-alanineamide.

As a result, 103 g/ℓ D-α-alanine (optical purity: 99.1%) was formed and accumulated at the end of the reaction.

Example 4

A desired mutant from Arthrobacter sp. H-4904 as the parent strain was obtained as follows.

The parent strain was cultured in an NB medium [a medium containing 20 g of bouillon powder (product of Kyokuto Co.) and 5 g of yeast extract (product of Difco Co.) in 1 ℓ of water, adjusted to pH 7.2 with NaOH] at 30°C for one day. The cells were recovered and suspended in a 0.1N Tris-maleic acid buffer solution (pH 6.0) at a concentration of 10^8 cells/mℓ, and then 400 μg/mℓ N-methyl-N′-nitro-N-

nitrosoguanidine (NTG) was added to the suspension. The suspension was incubated at room temperature for 30 minutes. The cells were thoroughly washed with physiological saline, smeared onto an NB agar medium (NB medium supplemented with 20 g of agar) and cultivated at 30°C for 1 to 6 days. The developing colonies were smeared on another NB agar medium and cultivated at 30°C for 1 to 2 days.

40 ml of a BYG medium in a 250 ml-Erlenmeyer flask which had been sterilized at 120°C for 20 minutes was inoculated with one loopful of the thus obtained strain and cultivated at 30°C for 21 hours with shaking. The thus obtained culture was centrifuged at 5,000 rpm for 10 minutes and the thus recovered cells were suspended in a 50 mM sodium phosphate buffer solution (pH 7.5) containing D-α-alanineamide hydrochloride to have a cell concentration of 2 g/ℓ as wet cells. The D-α-alanineamide hydrochloride concentration was adjusted to reach a final concentration of D-α-alanineamide of 25 g/ℓ. After reaction at 38°C for one hour, the thus formed D-α-alanine was quantitatively determined using high performance liquid chromatography under the same conditions as in Example 1 to calculate the D-amidase activity per gram of the wet cells.

The D-amidase activity was expressed as units per gram of wet cell. Arthrobacter sp. H-4904 was used as control and cultured in the same manner as above except that 2 g/ℓ DL-α-alanineamide was added to the medium, and the D-amidase activity was likewise determined.

In this manner, Arthrobacter sp. H-7095 was obtained as a mutant having the substantially same D-amidase activity as that of its parent strain, when cultured in a medium containing no inducing substance. The results are shown in Table 3.

Table 3

Strains	DL-α-Alanineamide	D-amidase activity (U/g wet cells)
Arthrobacter sp. H-4904	Added	666
Arthrobacter sp. H-7095	None	633

Example 5

The hydrolytic reaction was carried out in the same manner as in Example 2, except that cells of Arthrobacter sp. H-7095 were used which had been cultivated in a medium containing no DL-α-alanineamide.

The results are shown in Table 4.

Table 4

	Titer (g/ℓ)	Reaction yield (%)	Optical purity (%)
D-α-alanine	104	99	99.2
L-α-alanineamide	105	-	99.0

Example 6

The hydrolytic reaction was carried out in the same manner as in Example 5, except that 210 g of D-α-alanineamide was used in place of DL-α-alanineamide. As a result, 104 g/ℓ D-α-alanine (optical purity: 99.3%) was formed and accumulated at the end of reaction.

Example 7

The culturing was carried out using Arthrobacter sp. H-7095 in a similar manner as in Example 5 to

obtain 90 g of wet cells. The cells were washed with physiological saline and suspended in distilled water to make the total volume of 150 mℓ. A solution of 9 g of sodium arginate (Fuji Chemical Co., Ltd.) in 300 mℓ of distilled water was added to the suspension. The resulting mixture was dropwise added to 2% calcium chloride solution to give immobilized cells having a diameter of about 2 mm. Using the immobilized cells (25 g/ℓ), the reaction was carried out in the same manner as in Example 2. As the result, 105 g/ℓ D-α-alanine was formed and accumulated (optical purity: 98.2%).

Example 8

The culturing was carried out using Arthrobacter sp. H-7095 in a similar manner as in Example 5 to obtain 50 g of wet cells. The cells were suspended in 10 mM phosphate buffer (pH 7.0) to make the total volume of 200 mℓ. The cells contained in the suspension were disrupted with a homogenizer (Nissei Excel, Autohomogenizer) at 15,000 rpm for 20 minutes under ice-cooling. The resulting suspension was centrifuged at 4° C for 20 minutes at 12,000 rpm to give the supernatant as the cell extract. The supernatant (100 ml) was brought into contact with 20 mℓ of HPA-75 (product of Mitsubishi Kasei Corporation) which had been equilibrated with 10 mM phosphate buffer (pH 7.0) at 5° C for 24 hours. To the suspension of HPA-75 was added 0.5% glutaraldehyde solution, and the mixture was incubated at 4° C for 120 minutes to render HPA-75 crosslinked with D-amidase. Then, the mixture was washed 3 times with 10 mM phosphate buffer (pH 7.0) to give immobilized D-amidase. The specific activity of the immobilized product was 550 U/mℓ. Using the immobilized product (20 mℓ/ℓ), the reaction was carried out in the similar manner as in Example 2. As the result, 104 g/ℓ D-α-alanine was formed and accumulated (optical purity: 99.2%).

Claims

1. D-amidase having the following physicochemical properties:
(1) Activity and substrate specificity:
It specifically hydrolyzes D-α-alanineamide to D-α-alanine.
(2) Optimum pH:
pH 7 to 8 at 30° C
(3) Optimum temperature:
40 to 45° C at a pH of 7.5
(4) Heat stability:
It is inactivated when allowed to stand at a temperature above 60° C for 10 minutes.
(5) pH stability:
It is stable within a range of pH 6.5 to 10 at 30° C.
(6) Molecular weight:
50,000 ± 5,000 (SDS-polyacrylamide electrophoresis)
(7) Activation:
No coenzyme is required for activation.
(8) Isoelectric point:
pH 5.2 ± 0.3

2. A process for producing D-amidase defined by Claim 1, which comprises cultivating a microorganism belonging to the genus Arthrobacter and being capable of producing D-amidase, accumulating D-amidase in the culture and recovering D-amidase therefrom.

3. The process according to claim 2, wherein the medium contains DL-α-alanineamide, D-α-alanineamide or L-α-alanineamide as an inducing substance.

4. The process according to claim 2, wherein D-amidase is recovered from cells in the culture.

5. The process according to any one of Claims 2,3 and 4, wherein the microorganism is Arthrobacter sp. H-4904 (FERM BP-1649).

6. The process according to any one of Claims 2,3 and 4, wherein the microorganism is Arthrobacter sp. H-7095 (FERM BP-1773).

7. A process for producing D-α-alanine and/or L-α-alanineamide, which comprises carrying out an enzymatic hydrolysis in an aqueous medium containing DL-α-alanineamide or D-α-alanineamide in the presence of D-amidase defined by Claim 1, and recovering D-α-alanine and/or L-α-alanineamide from the resulting reaction mixture.

8. The process according to Claim 7, wherein the potency of D-amidase is 1 to 300 kU/ℓ.

9. The process according to Claim 7, wherein the aqueous medium contains 5 to 50% (v/v) glycerol.

10. The process according to Claim 7, wherein D-amidase is isolated from cells of a microorganism belonging to the genus Arthrobacter.

11. A process for producing D-α-alanine and/or L-α-alanineamide, which comprises carrying out an enzymatic hydrolysis in an aqueous medium containing DL-α-alanineamide or D-α-alanineamide in the presence of a culture or cells of a microorganism belonging to the genus Arthrobacter and being capable of producing D-amidase defined by Claim 1, or a treated product thereof, and recovering D-α-alanine and/or L-α-alanineamide from the resulting reaction mixture.

12. The process according to Claim 11, wherein the concentration of the cells is 1 to 50 g/ℓ as wet cells.

13. The process according to any one of Claims 7 and 11, wherein the concentration of D-α-alanineamide is 1 to 300 g/ℓ.

14. The process according to any one of Claims 7 and 11, wherein the concentration of DL-α-alanineamide is 1 to 500 g/ℓ.

15. The process according to any one of Claims 10 and 11, wherein the microorganism is Arthrobacter sp. H-4904 (FERM BP-1649).

16. The process according to any one of Claims 10 and 11, wherein the microorganism is Arthrobacter sp. H-7095 (FERM BP-1773).

17. A biologically pure culture of a microorganism of the genus Arthrobacter having the identifying characteristics of Arthrobacter sp. H-4904 (FERM BP-1649).

18. A biologically pure culture of a microorganism of the genus Arthrobacter having the identifying characteristics of Arthrobacter sp. H-7095 (FERM BP-1773).

FIG.1

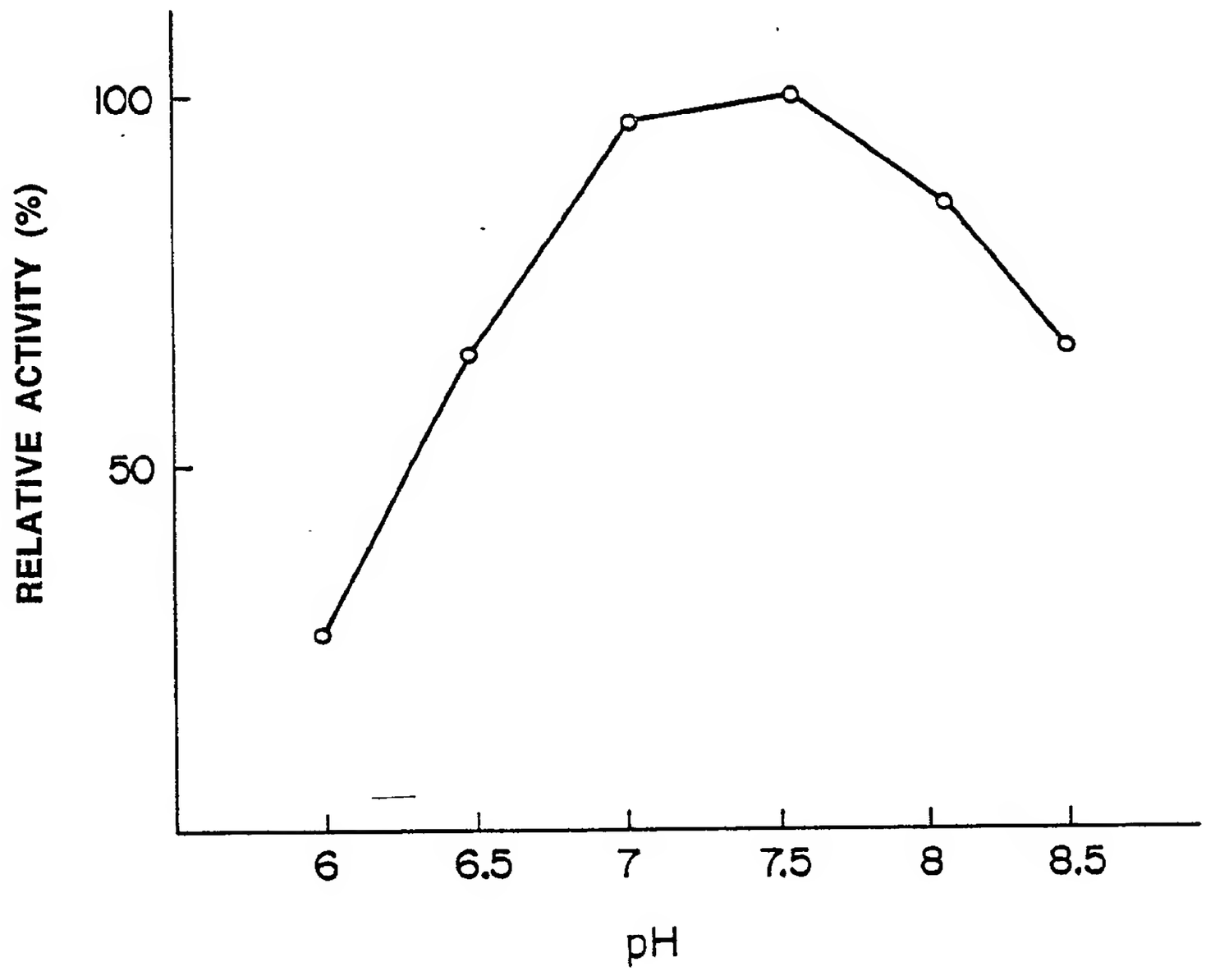


FIG.2

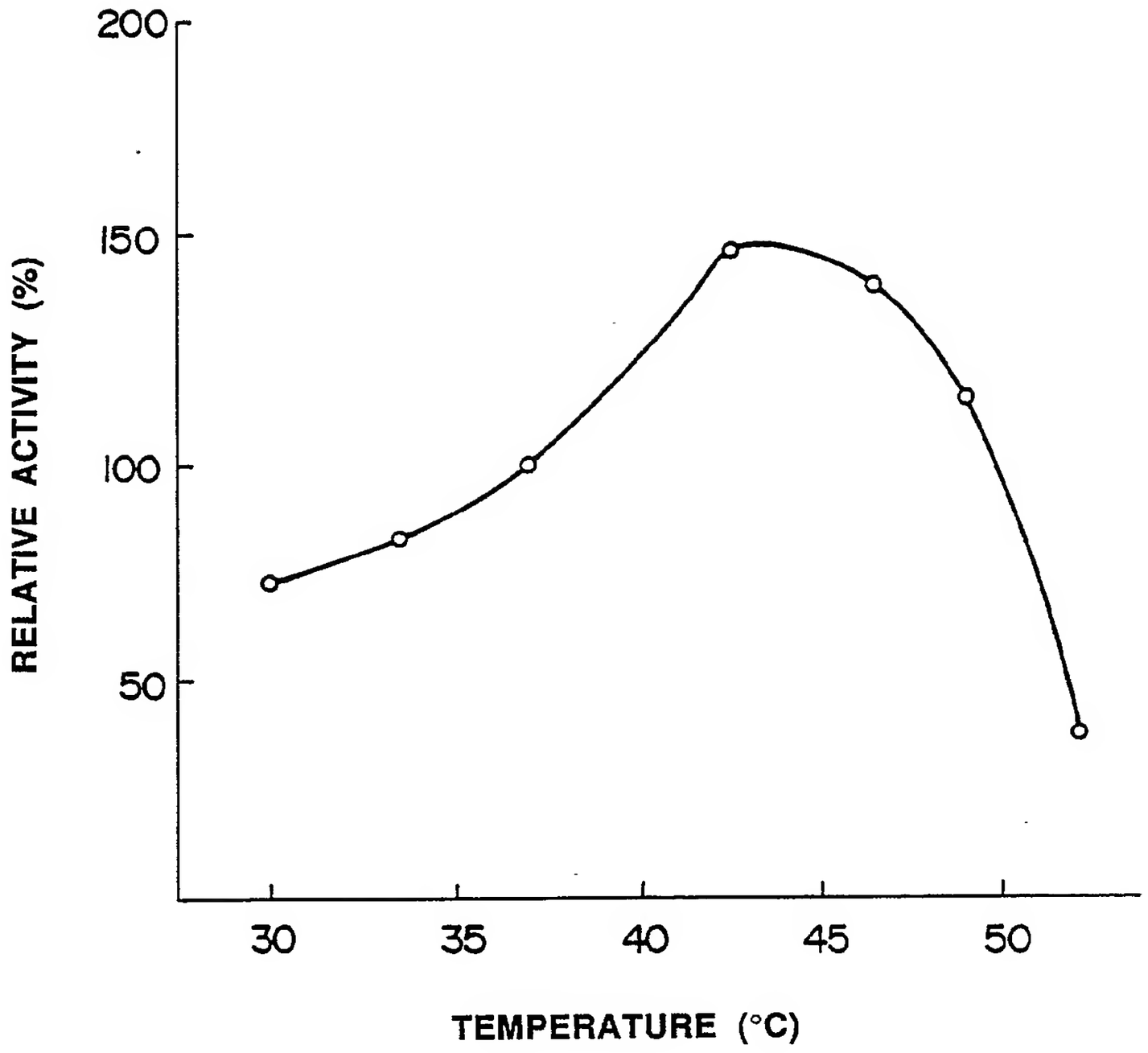


FIG.3

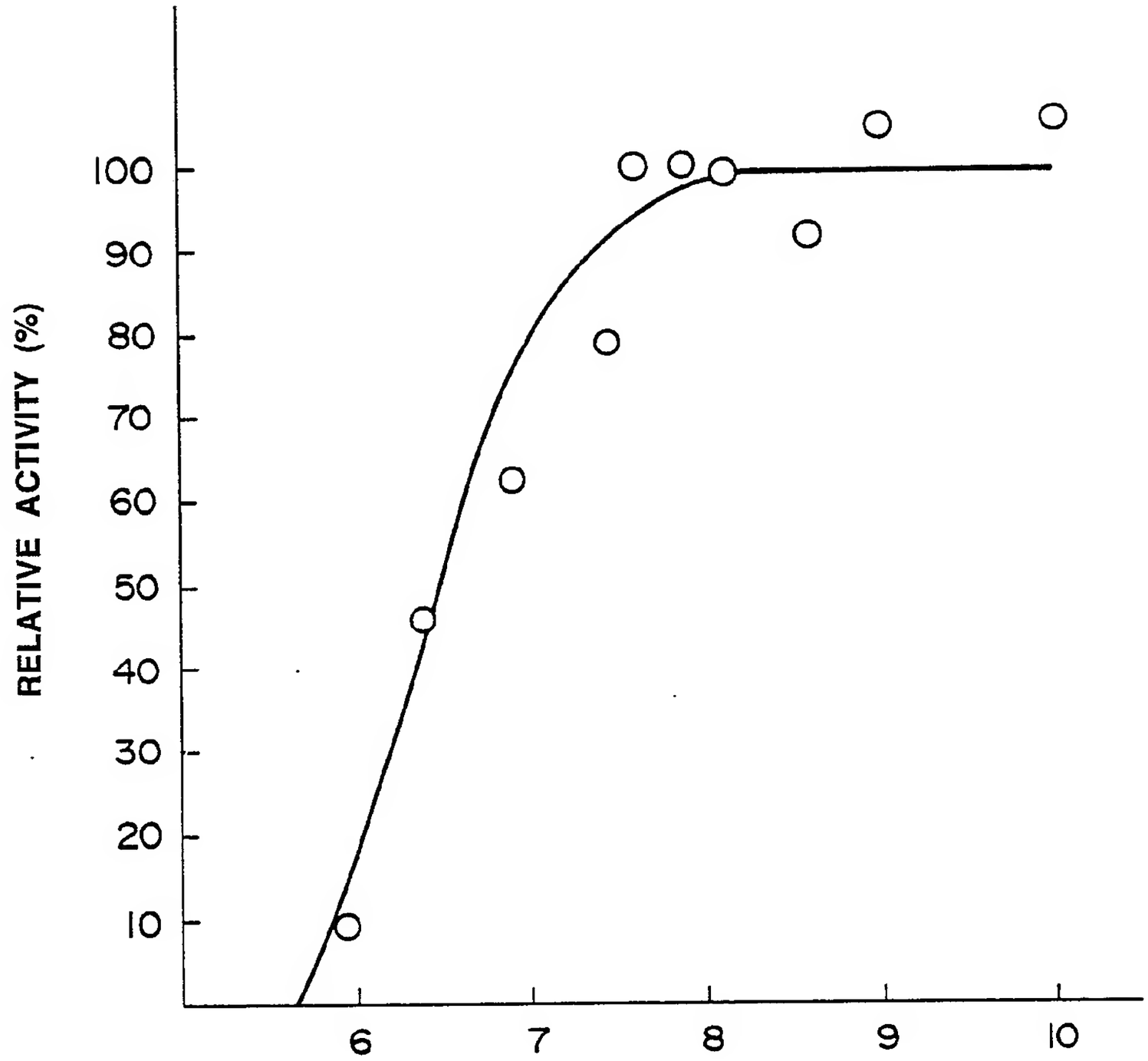
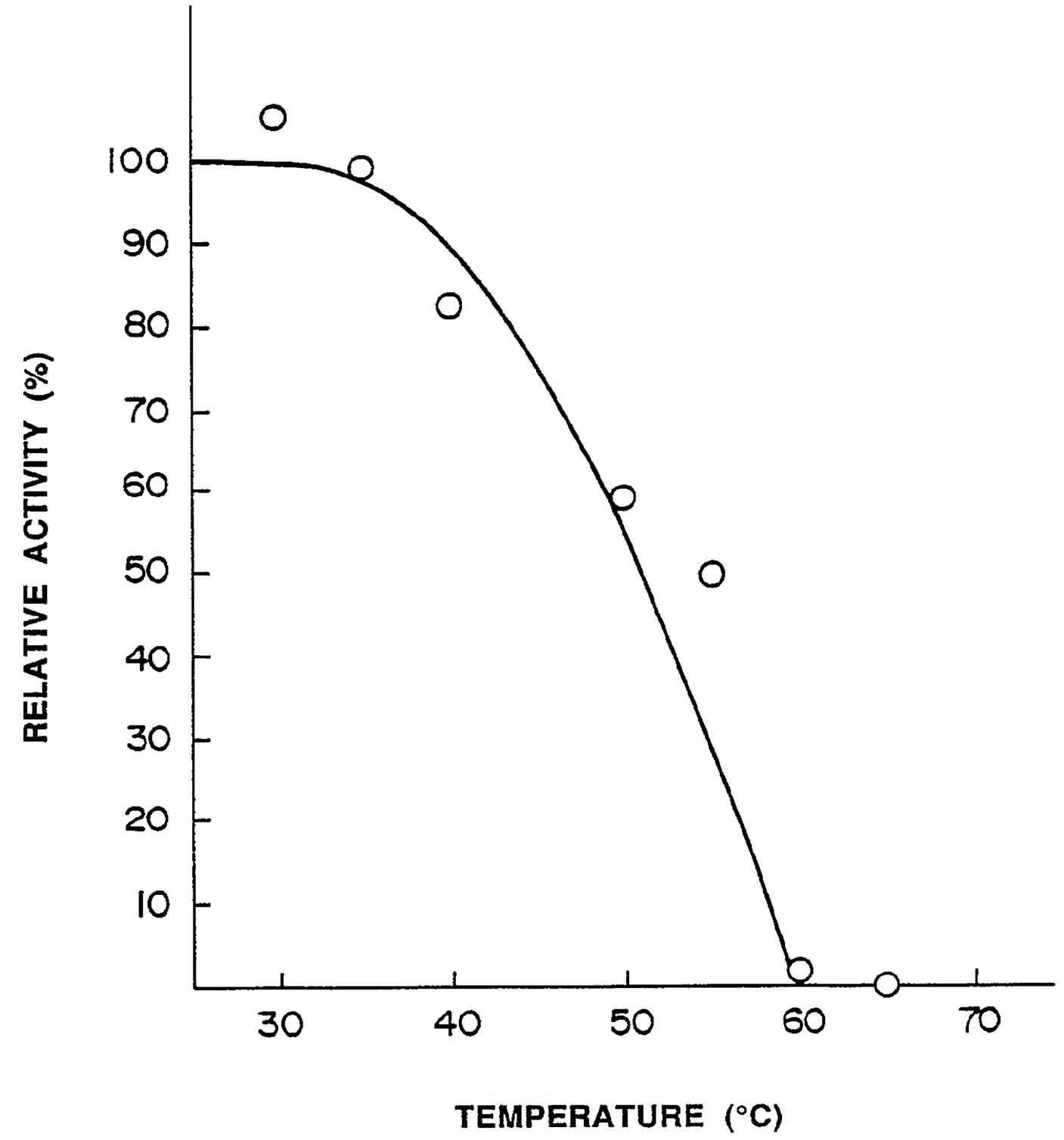


FIG. 4


RELATIVE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
30
40
50
60
70
TEMPERATURE (°C)